PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12N 15/45, C07K 14/115, 16/10, A61K 39/155, G01N 33/569 — A1

(11) International Publication Number: **WO 00/20600**

(43) International Publication Date: 13 April 2000 (13.04.00)

(21) International Application Number: PCT/EP99/07400

(22) International Filing Date: 29 September 1999 (29.09.99)

(30) Priority Data:
98203352.4 5 October 1998 (05.10.98) NL

(71) Applicant *(for all designated States except US)*: AKZO NOBEL N.V. [NL/NL]; Velperweg 76, NL–6824 AB Arnhem (NL).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: COOK, Jane, Kathleen, Alexandra [GB/GB]; 138 Hartford Road, Huntingdon, Cambs PE187 XQ (GB). TARPEY, Ian [GB/GB]; 12 The Quadrant, St. Ives, Cambridge PE17 4PE (GB). HUGGINS, Michael, Brian [GB/GB]; 29 Humberdale Way, Warboys, Huntingdon, Cambs PE17 2RX (GB). DAVIS, Phil [GB/GB]; 10 Laburnum Way, St. Ives, Huntingdon, Cambs PE17 6YW (GB).

(74) Agent: MESTROM, J., J., L.; P.O. Box 20, NL–5340 BH OSS (NL).

(81) Designated States: AL, AU, BA, BB, BG, BR, CA, CN, CU, CZ, EE, GE, HU, ID, IL, IN, IS, JP, KP, KR, LC, LK, LR, LT, LV, MG, MK, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, SL, TR, TT, UA, US, UZ, VN, YU, ZA, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: AVIAN PNEUMOVIRUS VACCINE AND DIAGNOSTIC AGENT

(57) Abstract

The invention relates to a nucleic acid sequence encoding an avian pneumovirus related polypeptide. The avian pneumovirus related protein encoded by the nucleic acid sequence can be used for the preparation of a vaccine against APV or for diagnostic purposes. The invention is also concerned with a test kit useful for the detection of APV infected poultry.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

Avian pneumovirus vaccine & diagnostic agent

The present invention is concerned with a nucleic acid sequence encoding an avian pneumovirus related polypeptide, a recombinant nucleic acid molecule comprising such a nucleic acid sequence, a recombinant vector virus comprising said nucleic acid sequence, a host cell transformed with such a nucleic acid sequence, an avian pneumovirus related polypeptide encoded by the nucleic acid sequence and antibodies reactive with the polypeptide as well as a vaccine against avian pneumovirus infection.

The invention also relates to an immunochemical reagent and a test kit comprising said reagent.

Avian pneumoviruses form one of the two genera of the Pneumoviridae, which in turn is one of the two subfamilies belonging to the family Paramyxoviridae. The Paramyxoviridae are members of the Order Mononegavirales, which are viruses containing nonsegmented, negative sense, single-stranded RNA.

The first avian pneumovirus to be described was turkey rhinotracheitis (TRT) virus (Cavanagh D. & Barrett T., Virus Research, 11, 241-256, [1988]). This virus causes a highly contagious upper respiratory tract infection in turkeys resulting in high morbidity and variable mortality. In laying turkeys, TRT virus can cause a substantial drop in egg production and poor shell quality.

TRT virus has also been incriminated as a pathogen in chickens, and TRT infection has also been demonstrated in pheasants and guinea fowl.

Turkey rhinotracheitis was first described in South Africa in the late 1970s, where it had a devastating effect on the turkey industry. Within a few years TRT had spread to Israel and parts of Europe. It is now a major disease threat to the poultry industry worldwide. TRT infections have continued to be reported in many countries, but until now, not in North America.

(Senne, D. A. *et al*, Proc. Amer. Vet. Med. Assoc. 134th Annual Congress, Reno, Nevada, July 1997, p190).

Using neutralisation tests in tracheal organ cultures (TOC), all TRT isolates studied from many countries, and obtained from both turkeys and chickens, have been found to belong to a single serotype (Cook, J.K.A. *et al*, [1993], Avian Pathology, 22, 257-273). TRT virus has two surface glycoproteins, F and G, which are important for the induction of protective immunity. Based on the results of both neutralisation tests in TOC, using monoclonal antibodies which recognise the G glycoprotein and molecular sequencing of the G glycoprotein (Juhasz & Easton, 1994, Journal of General Virology, 75, 2873-2880), two subgroups, A and B, within the single serotype have been recognised.

Following isolation in tracheal organ cultures, serial passage of TRT virus in a variety of cell cultures, including chick embryo fibroblasts and VERO cells, has resulted in rapid attenuation of the virus.

Current TRT vaccines comprise chemically inactivated or live attenuated viruses (Cook, J.K.A. *et al*, [1989] Avian Pathology, 18, 511-522 & 523-534; Williams, R.A., [1991], Avian Pathology, 20, 45-55 & 585-596; Cook, J.K.A., [1996], Avian Pathology, 25, 231-243). Inactivated vaccines, however, require additional immunizations, disadvantageously contain adjuvants, are expensive to produce and are laborious to administer. Furthermore, some infectious particles may survive the inactivation process and may cause disease after administration to the bird.

In general, attenuated live virus vaccines are preferred because they evoke an immune response often based on both humoral and cellular reactions. Up to now, such vaccines based on TRT strains can only be prepared by serial passage of virulent strains in cell culture. However, because of this treatment uncontrolled mutations can be introduced into the viral genome, resulting in a population of virus particles heterogeneous with regard to virulence and immunizing properties. In addition, it is well known that such traditional attenuated live virus vaccines can revert to virulence resulting in disease outbreaks in inoculated birds and the possible spread of the pathogen to other birds. Using recombinant DNA technology, a fowl pox

vector expressing the F gene of TRT virus has been demonstrated to protect turkeys against homologous challenge (Yu, Q. *et al*, [1994], Vaccine, 12, 569-573), but this approach has not been used commercially.

In 1996 a disease in turkeys was reported in the State of Colorado, USA, which was characterised by coughing, sneezing and tracheal rales. Clinically the disease appeared to be similar to TRT as seen in other countries. Examination of sera taken from infected birds has revealed a low incidence of antibodies to TRT virus when tested by ELISA.

The causal agent of the Colorado outbreak was isolated from swabs collected from the infected turkeys by passaging the material 3 times via the yolk sac of embryonated chicken eggs, followed by several passages in chick embryo fibroblasts (Senne, D. A. *et al*, Proc. Amer. Vet. Med. Assoc. 134th Annual Congress, Reno, Nevada, July 1997, p190). The cytopathic effect seen was characterised by syncytial and giant cell formation. Preliminary characterisation as a pneumovirus was done using a TRT fluorescent antibody.

The Colorado isolate was compared to known TRT strains in *in vitro* serum neutralisation tests. First, the Colorado isolate was tested against monospecific polyclonal antisera to recognised TRT isolates. The Colorado isolate was not neutralised by monospecific antisera to any of the TRT strains. It was, however, neutralised by a hyperimmune antiserum raised against a subgroup A strain. This antiserum neutralised the homologous virus to a titre of 1:400 and the Colorado isolate to a titre of 1:80.

Using the above method, the Colorado isolate was then tested against TRT monoclonal antibodies (Cook *et al.*, 1993, Avian Pathology, 22, 257-273). In each case, the reciprocal neutralisation titre was <10.

Monospecific antiserum raised to the Colorado isolate was also tested against TRT strains of both subgroups. None of the TRT strains tested were neutralised by the antiserum to the Colorado isolate.

By inoculating specific pathogen free (SPF) chickens via the oculonasal route with viral inoculum, and subsequently challenging the birds with either a TRT virus strain subgroup A or a TRT virus strain subgroup B it has been found that the Colorado strain of avian pneumovirus does not protect SPF chicks against challenge with either a subgroup A or a subgroup B strain of TRT virus.

This evidence, together with results of the neutralisation tests in which the Colorado isolate and TRT strains of both subgroups A and B were compared, suggests that the Colorado isolate may be the first example of a second serotype of avian pneumovirus.

A vaccine against this new serotype of avian pneumovirus might be constructed based on recombinant DNA technology. Such a vaccine would only contain the necessary and relevant avian pneumovirus immunogenic material which is capable of eliciting a protective response against the Colorado strain, or the genetic information encoding said material, and would not display the above-mentioned disadvantages of live or inactivated TRT vaccines.

According to the present invention there is provided a nucleic acid sequence encoding a polypeptide comprising at least part of the protein expressed by the F gene of an avian pneumovirus characterised in that the polypeptide has an amino acid sequence shown in SEQ. ID No. 2 or is a functional variant thereof.

"Nucleic acid sequence" as used herein refers to a polymeric form of nucleotides of any length, both to ribonucleic acid sequences and to deoxyribonucleic acid sequences. In principle, this term refers to the primary structure of the molecule. Thus, this term includes double and single-stranded DNA, as well as double and single-stranded RNA and modifications thereof.

In general, the term "polypeptide" refers to a molecular chain of amino acids with a biological activity, it does not refer to a specific length of the product and if required can be modified *in vivo* or *in vitro*, for example by glycosylation, amidation, carboxylation or phosphorylation; thus inter dia, peptides, oligopeptides and proteins are included.

SEQ. ID. No. 1 shows the nucleotide sequence of the Colorado isolate APV F gene (this sequence is given in the mRNA sense) and the amino acid sequence of the Colorado isolate APV F protein.

SEQ. ID. No. 2 shows the amino acid sequence of the Colorado isolate APV F protein encoded by the gene shown in SEQ. ID. No. 1.

Also included within the scope of the present invention are nucleic acid sequences which encode a fuctional variant of the polypeptide having the amino acid sequence shown in SEQ. ID. No. 2. Such functional variant polypeptides exhibit at least 80% homology with said sequence, preferably at least 90% homology, most preferably at least 95% homology, and are able to induce antibodies in poultry which react with the polypetide shown in SEQ. ID. No. 2

Several techniques are bred routinely to clone, sequence and express the F gene from the Colorado isolate of avian pneumovirus. Such molecular biology methods are standard and are described in Molecular Cloning. A Laboratory Manual. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Cold Spring Harbour, New York, and also in Current Protocols in Molecular Biology (Eds. Anfubel, Brent, Kingston, More, Friedman, Smith and Stuhl, Greene Publishing Associates; Wiley Interscience, NY (1992). These include making cDNA from isolated mRNA, performing *in vitro* mRNA translations and several ways of transcript mapping, like S1 nuclease-protection assays and primer extension analyses.

It will be understood that for the particular polypeptide shown in SEQ. ID. No. 2, derived from the Colorado isolate of APV, natural variations can exist between individual viruses. These variations may be demonstrated by (an) amino acid difference(s) in the overall sequence or by deletions, substitutions, insertions, inversions or additions of (an) amino acid(s) in said sequence. Amino acid substitutions from which can be expected that they do not essentially alter biological and immunological activites, have been described. Amino acid replacements between related amino acids or replacements which have occurred frequently in evolution are, inter alia Ser/Ala, Ser/Gly, Asp/Gly, Asp/Asn, Ile/Val (see Dayhof, M.D., Atlas of protein sequence and structure, Nat. Biomed. Res. Found., Washington D.C., 1978, vol 5, suppl. 3). Based on this information Lipman and Pearson developed a method for rapid and sensitive

protein comparison (Science 227, 1435-1441, 1985) and determining the functional similarity between homologous polypeptides. Nucleic acid sequences encoding such homologous functional equivalents to the F protein specifically disclosed herein are included within the scope of this invention. Moreover, the potential exists to use recombinant DNA technology of the preparation of nucleic acid sequences encoding these various functional equivalents.

The information provided in SEQ. ID. Nos. 1 and 2 allows a person skilled in the art to isolate and identify nucleic acid sequences encoding the various functional variant polypeptides mentioned above having corresponding immunological characteristics with the Colorado isolate APV protein specifically disclosed herein. The generally applied Southern Blotting technique or colony hybridization can be used for that purpose (Experiments in Molecular Biology, ed. R.J. Slater, Clifton, U.S.A., 1986); Singer-Sam, J. *et al*, Proc. Natl. Acad. Sci. 80, 802-806, 1983; Maniatis T. *et al*, Molecular Cloning, A Laboratory Manual, Cold Spring Harbour Laboratory Press, 1989). For example, restriction enzyme digested DNA fragments derived from the Colorado isolate APV is electrophoresed and transferred, or "blotted" thereafter onto a piece of nitrocellulose filter. It is now possible to identify the nucleic sequences encoding the functional variant polypeptides on the filter by hybridization to a defined labelled DNA or "probe" back translated from the amino acid sequence shown in SEQ. ID. No. 2 under specific conditions of salt concentration and temperature that allow hybridization of the probe to any homologous DNA sequences present on the filter. After washing the filter, hybridized material may be detected by autoradiography. Once having identified the relevant sequence, DNA fragments that encode a functionally variant polypeptide to the polypeptide disclosed in SEQ. ID. No. 2 can now be recovered after agarose gel electrophoresis by elution and used to direct the synthesis of a polypeptide functionally equivalent to a polypeptide disclosed in SEQ. ID. No. 2.

In another way APV cDNA may be cloned into a phage such as λgt 11 and expressed in a bacterial host. Recombinant phages can then be screened with polyclonal serum raised against the purified Colorado isolate APV polypeptide disclosed in SEQ. ID. No. 2, determining the presence of corresponding regions of the variant polypeptide.

As is well known in the art, the degeneracy of the genetic code permits substitution of bases in a codon resulting in another codon but still coding for the same amino acid e.g. the codon for the amino acid glutamic acid is both GAT and GAA. Consequently, it is clear that for the expression of a polypeptide with the amino acid sequence shown in SEQ. ID. No. 2 use can be made of a derivative nucleic acid sequence with such an alternative codon composition different from the nucleic acid sequence shown in SEQ. ID. No. 1.

In addition, fragments of the nucleic acid sequence encoding the Colorado isolate APV polypeptide or functional equivalents thereof as mentioned above are included in the present invention.

The term "fragment" as used herein means a DNA or amino acid sequence comprising a subsequence of a nucleic acid sequence or polypeptide defined above. Said fragment is or encodes a polypeptide having one or more immunogenic determinants of the Colorado isolate APV polypeptide defined above, i.e. has one or more epitopes of the APV polypeptide which are capable of inducing antibodies in poultry which react with the polypetide disclosed in SEQ. ID. No. 2 to a greater extent as with the F proteins of subgroup A and B TRT viruses. Methods for determining usable polypeptide fragments are outlined below. Fragments can inter alia be produced by enzymatic cleavage of precursor molecules, using restriction endonucleases for the DNA and proteases for the polypeptides. Other methods include chemical synthesis of the fragments or the expression of polypeptide fragments by DNA fragments.

A nucleic acid sequence according to the present invention can be ligated to various replication effecting DNA sequences with which it is not associated or linked in nature resulting in a so called recombinant vector molecule which can be used for the transformation of a suitable host. Useful recombinant vector molecules, are preferably derived from, for example plasmids, bacteriophages, cosmids or viruses.

Specific vectors or cloning vehicles which can be used to clone nucleic acid sequences according to the invention are known in the art and include inter alia plasmid vectors such as pBR322, the various pUC, pGEM and Bluescript plasmids, bacteriophages, e.g. λgt-Wes-λB, Charon 28 and the M13 derived phages or viral vectors such as SV40, adenovirus or polyoma

virus (see also Rodriquez, R.L. and D.T. Denhardt, ed., Vectors: A survey of molecular cloning vectors and their uses, Butterworths, 1988; Lenstra, J.A. *et al.*, Arch. Virol. 110, 1-24, 1990). The methods to be used for the construction of a recombinant vector molecule according to the invention are known to those of ordinary skill in the art and are inter alia set forth in Maniatis, T. *et al* (Molecular Cloning A Laboratory Manual, second edition; Cold Spring Harbor Laboratory 1989).

Alternatively, the APV cDNA may be inserted into a recombinant virus vector such as turkey herpesvirus (HVT), infectious laryngeotracheitis (ILT) or Fowlpox. This can be achieved by cloning the sequence into the vector downstream from a promoter to drive expression. Such a vector would also contain flanking regions homologous to the virus into which it is to be inserted. Co-transfection of suitable cells with the vector and viral DNA can lead to homologous recombination so that a proportion of viruses contain the inserted gene.

In yet another alternative the APV cDNA may be inserted into a DNA vaccination vector such as pI.18 which may then be used directly for vaccination.

For example, the insertion of the nucleic acid sequence according to the invention into a cloning vector can easily be achieved when both the genes and the desired cloning vehicle have been cut with the same restriction enzyme(s) as complementary DNA termini are thereby produced.

Alternatively, it may be necessary to modify the restriction sites that are produced into blunt ends either by digesting the single-stranded DNA or by filling in the single-stranded termini with an appropriate DNA polymerase. Subsequently, blunt end ligation with an enzyme such as T4 DNA ligase may be carried out.

If desired, any restriction site may be produced by ligating linkers onto the DNA termini. Such linkers may comprise specific oligonucleotide sequences that encode restriction site sequences. The restriction enzyme cleaved vector and nucleic acid sequence may also be modified by homopolymeric tailing.

"Transformation", as used herein, refers to the introduction of a heterologous nucleic acid sequence into a host cell, irrespective of the method used, for example direct uptake or transduction. The heterologous nucleic acid sequence may be maintained through autonomous replication or alternatively, may be integrated into the host genome. If desired, the recombinant vector molecules are provided with appropriate control sequences compatible with the designated host which can regulate the expression of the inserted nucleic acid sequence. In addition to micro-organisms, culture of cells derived from multicellular organisms may also be used as hosts.

The recombinant vector molecules according to the invention preferably contain one or more marker activities that may be used to select for desired transformants, such as ampicillin and tetracycline resistance in pBR322, ampicillin resistance and β-galactosidase activity in pUC8.

A suitable host cell is a pro- or eukaryotic cell which can be transformed by a nucleic acid sequence encoding a polypeptide or by a recombinant vector molecule comprising such a nucleic acid sequence and which can if desired be used to express said polypeptide encoded by said nucleic acid sequence. The host cell can be of prokaryotic origin, e.g. bacteria such as *Escherichia coli*, *Bacillus subtilis* and *Pseudomonas* species; or of eukaryotic origin such as yeasts, e.g. Saccharomyces cerevisiae or higher eukaryotic cells such as insects, plant or mammalian cells, including HeLa cells and Chinese hamster ovary (CHO) cells. Insects cells include the Sf9 cell line of *Spodoptera frugiperda* (Luckow *et al.*, Bio-technology 6, 47-55, 1988). Information with respect to the cloning and expression of the nucleic acid sequence of the present invention in eukaryotic cloning systems can be found in Esser, K. *et al.* (Plasmids of Eukaryotes, Springer-Verlag, 1986).

In general, prokaryotes are preferred for cloning of DNA sequences in constructing the vectors useful in the invention. For example E.coli K12 and derivative strains such as DH5α or JM101 are particularly useful.

For expression, nucleic acid sequences of the present invention are introduced into an expression vector, such as yeast, (baculo) virus or bacterial systems. Such sequences are

operably linked to expression control sequences which may comprise promoters, enhancers, operators, inducers, ribosome binding sites etc. Therefore, the present invention provides a recombinant vector molecule comprising a nucleic acid sequence encoding the Colorado isolate APV polypeptide as defined above operably linked to expression control sequences, capable of expressing the DNA sequences contained therein in (a) transformed host cell(s).

It should, of course, be understood that the nucleotide sequences inserted at the selected site of the cloning vector may include nucleotides which are not part of the actual structural gene for the desired polypeptide or may include only a fragment of the complete structural gene for the desired protein as long as transformed host will produce a polypeptide having at least one or more immunogenic determinants of the Colorado isolate APV polypeptide as defined above.

When the host cells are bacteria, illustrative useful expression control sequences include the Trp promoter and operator (Goeddel, *et al.*, Nucl. Acids Res. 8, 4057, 1980); the lac promoter and operator (Chang, *et al.*, Nature 275, 615, 1978); the outer membrane protein promoter (Nakamura, K. and Inouge, M., EMBO J. 1, 771-775, 1982); the bacteriophage λ promoters and operators (Remaut, E. *et al.*, Nucl. Acids Res. 11, 4677-4688, 1983); the α-amylase (B. subtilis) promoter and operator, termination sequence and other expression enhancement and control sequences compatible with the selected host cell. When the host cell is yeast, illustrative useful expression control sequences include, e.g., α-mating factor. For insect cells the polyhedrin or p10 promoters of baculoviruses can be used (Smith, G. E. *et al.*, Mol. Cell. Biol, 3, 2156-65, 1983). When the host cell is of mammalian origin illustrative useful expression control sequences include, e.g. the SV40 promoter (Berman, P.W. *et al.*, Science 222, 524-527, 1983) or, e.g. the metallothionein promoter (Brinster, R.L., Nature 296, 39-42, 1982) or a heat shock promoter (Voellmy *et al.*, Proc. Natl. Acad. Sci. USA 82, 4949-53, 1985). Alternatively, also expression control sequences present in APV, may be applied. For maximizing gene expression, see also Roberts and Leuer (Methods in Enzymology 68, 473, 1979).

Therefore, the invention also comprises (a) host cell(s) transformed with a nucleic acid sequence or recombinant expression vector molecule described above, capable of producing the Colorado isolate APV polypeptide as defined above by expression of the necleic acid sequence.

The present invention also provides a purified polypeptide having one or more immunogenic determinants of an APV protein having an amino acid sequence shown in SEQ. ID. No. 2, or a functional variant thereof, essentially free from the whole virus or other protein with which it is ordinarily associated.

It will be understood that derivatives of said amino acid sequences displaying the same immunological properties in essence, i.e. functional variants, are also within the scope of the present invention.

In addition a polypeptide which can be used for immunization of poultry against APV infection or disease or for diagnostic purposes substantially comprising an immunogenic fragment of the Colorado isolate APV polypeptide is included in the present invention. Various methods are known for detecting such usable polypeptide fragments within a amino acid sequence.

Suitable immunochemically active immunogenic fragments of a polypeptide according to the invention containing (an) epitope(s) can be found by means of the method described in Patent Application WO 86/06487, Geysen, H.M. *et al.* (Prod. Natl. Acad. Sci. 81, 3998-4002, 1984), Geysen, H.M. *et al.* (J. Immunol. Meth. 102, 259-274, 1987) based on the so-called pep-scan method, wherein a series of partially overlapping polypeptides corresponding with partial sequences of the complete polypeptide under consideration, are synthesized and their reactivity with antibodies is investigated.

In addition, a number of regions of the polypeptide, with the stated amino acid sequence, can be designated epitopes on the bases of theoretical considerations and structural agreement with epitopes which are now known. The determination of these regions is based on a combination of the hydrophilicity criteria according to Hopp and Woods (Proc. Natl. Acad. Sci. 78, 3824-3828, 1981) and the secondary structure aspects according to Chou and Fasman (Advances in Enzymology 47, 45-148, 1987)

T-cell epitopes which may be necessary can likewise be derived on theoretical grounds with the aid of Berzofsky's amphiphilicity criterion (Science 235, 1059-62, 1987).

In another embodiment of the invention a polypeptide having an amino acid sequence encoded by a nucleic acid sequence mentioned above is used.

Immunization of poultry against APV infection or disease can, for example be achieved by administering to the birds a polypeptide according to the invention in an immunologically relevent context as a so-called subunit vaccine. The subunit vaccine according to the invention may comprise a polypeptide in a pure form, optionally in the presence of a pharmaceutically acceptable carrier. The polypeptide can optionally be covalently bonded to a non-related protein, which, for example can be of advantage in the purification of the fusion product. Examples are β-galactosidase, protein A, prochymosine, blood clotting factor Xa, etc.

In some cases the ability to raise neutralizing antibodies against these polypeptides per se may be low. Small fragments are preferably conjugated to carrier molecules in order to raise their immunogenicity. Suitable carriers for this purpose are macromolecules, such as natural polymers (proteins like key hole limpet haemocyanin, albumin, toxins), synthetic polymers like polyamino acids (polylysine, polyalanine), or micelles of amphiphilic compounds like saponins. Alternatively these fragments may be provided as polymers thereof, preferably linear polymers.

Polypeptides to be used in such subunit vaccines can be prepared by methods know in the art, e.g. by isolating said polypeptides from APV by recombinant DNA techniques or by chemical syntheses.

If required the polypeptides according to the invention to be used in a vaccine can be modified *in vitro* or *in vivo*, for example by glycosylation, amidation, carboxylation or phosphorylation.

An alternative to subunit vaccines are live vector vaccines. A nucleic acid sequence according to the invention is introduced by recombinant DNA techniques into a micro-organism (e.g. a bacterium or virus) in such a way that the recombinant micro-organism is still able to

replicate thereby expressing a polypeptide coded by the inserted nucleic acid sequence and eliciting an immune response in the infected host animal.

A preferred embodiment of the present invention is a recombinant vector virus comprising a heterologous nucleic acid sequence described above, capable of expressing the DNA sequence in (a) host cell(s) or host animal infected with the recombinant vector virus. The term "heterologous" indicates that the nucleic acid sequence according to the invention is not normally present in the nature in the vector virus or is not present in the same genetic context as in the naturally occurring vector virus.

Furthermore, the invention also comprises (a) host cell(s) or cell culture infected with the recombinant vector virus, capable of producing the APV protein by expression of the nucleic acid sequence.

The well know technique of *in vivo* homologous recombination can be used to introduce a heterologous nucleic acid sequence, e.g. a nucleic acid sequence according to the invention into the genome of the vector virus.

First, a DNA fragment corresponding with an insertion region of the vector genome, i.e. a region which can be used for the incorporation of a heterologous sequence without disrupting essential functions of the vector such as those necessary for infection or replication, is inserted into a cloning vector according to standard recDNA rechniques. Insertion-regions have been reported for a large number of micro-organisms (e.g. EP 80,806, EP 110,385, EP 83,286, EP 314,569, WO88/07088).

Second, if desired, a deletion can be introduced into the insertion region present in the recombinant vector molecule obtained from the first step. This can be achieved for example by appropriate exonuclease lll digestion or restriction enzyme treatment of the recombinant vector molecule from the first step.

Third, the heterologous nucleic acid sequence is inserted into the insertion-region present in the recombinant vector molecule of the first step or in place of the DNA deleted from said recombinant vector molecule. The insertion region DNA sequence should be of appropriate length as to allow homologous recombination with the vector genome to occur. Thereafter, suitable cells can be infected with wild-type vector virus or transformed with vector genomic DNA in the presence of the recombinant vector molecule containing the insertion flanked by appropriate vector DNA sequences whereby recombination occurs between the corresponding regions in the recombinant vector molecule and the vector genome. Recombinant vector progeny can now be produced in cell culture and can be selected for example genotypically or phenotypically, e.g. by hybridization, detecting enzyme activity encoded by a gene co-integrated along with the heterologous nucleic acid sequence, or detecting the antigenic heterologous polypeptide expressed by the recombinant vector immunologically.

Next, this recombinant micro-organism can be administered to the host animal for immunization after which it maintains itself for some time, or even replicates in the body of the inoculated animal, expressing *in vivo* a polypeptide coded for by the inserted nucleic acid sequence according to the invention resulting in the stimulation of the immune system of the inoculated animal. Suitable vectors for the incorporation of a nucleic acid sequence according to the invention can be derived from viruses such as (avian) poxviruses, e.g. vaccinia virus or fowl poxvirus (EP 314,569 and WO 88/02022), herpesviruses, for example vaccine strains of Marek's Disease viruses such as HVT (WO 88/07088) or MDV strains of serotype 1 or 2, adenovirus or influenza virus, or bacteria such as *E. coli* or specific *Salmonella* species. These vectors can be designed in a way to obtain an efficient recognition of the polypeptide by the immune system of the host animal. In this context fusion of the said polypeptide with synthetic signal and anchor sequences or with an antigen from another viral or bacterial pathogen, e.g. the haemagglutinin-neuraminidase protein form Newcastle disease virus, are conceivable. It is also possible that the said immunogenic polypeptide, if desired as part of a larger whole, is released inside the animal to be immunized. In all of these cases it is also possible that one or more immunogenic products will find expression which generate protection against various pathogens and/or against various antigens of a given pathogen.

A vaccine according to the invention can be prepared by culturing a host cell infected with a recombinant vector virus comprising a nucleic acid sequence according to the invention, after which virus containing cells and/or recombinant vector viruses grown in the cells can be collected, optionally in a pure form. Another possibility to produce a vector vaccine is to incorporate a nucleic acid sequence according to the invention in an infectious bacteria or parasite and to culture such a modified infectious agent. After collection, optionally in a pure form, a vaccine, optionally in a lyophilized form, can be produced.

Host cells transformed with a recombinant vector molecule according to the invention can also be cultured under conditions which are favourable for the expression of a polypeptide coded by said nucleic acid sequence. Vaccines may be prepared using samples of the crude culture, host cell lysates or host cell extracts, although in another embodiment more purified polypeptides according to the invention are formed to a vaccine, depending on its intended use. In order to purify the polypeptides produced, host cells transformed with a recombinant vector according to the invention are cultured in an adequate volume and the polypeptides produced are isolated from such cells or from the medium if the protein is excreted. Polypeptides excreted into the medium can be isolated and purified by standard techniques, e.g. salt fractionation, centrifugation, ultrafiltration, chromatography, gel filtration or immuno affinity chromatography, whereas inter cellular polypeptides can be isolated by first collecting said cells, disrupting the cell, for example by sonication or by other mechanically disruptive means such as French press followed by separation of the polypeptides from the other inter cellular components and forming the polpeptides to a vaccine. Cell disruption could also be accomplished by chemical (e.g. EDTA treatment) or enzymatic means such as lysozyme digestion.

Antibodies or antiserum directed against a polypeptide according to the invention have potential use in passive immunotherapy, diagnostic immunoassays and generation of anti-idiotype antibodies.

The Colorado isolate APV polypeptide as characterized above can be used to produce antibodies by methods well known in the art.

The vaccine according to the invention can be administered in a conventional active immunization scheme: single or repeated administration in a manner compatible with the dosage formulation and in such amount as will be prophylactically and/or therapeutically effective and immunogenic, i.e. the amount of immunizing antigen or recombinant micro-organism capable of expressing said antigen that will induce immunity in an animal against challenge by a virulent APV. Immunity is defined as the induction of a higher level of protection in a population of animals after vaccination compared to an unvaccinated group.

For live viral vector vaccines the dose rate per animal may range from 1.10^2 - 1.10^6 infectivity units.

A typical subunit vaccine according to the invention comprises 10µg - 1mg of the polypeptide according to the invention.

The administration of the vaccine can be done, e.g. intradermally, subcutaneously, intramuscularly, *in ovo* via spray or drinking water or by 'biolistic device' such as a gene gun, intraperitonially, intravenously, by eye-drop, orally or intranasally.

Additionally the vaccine may also contain an aqueous medium or a water containing suspension, often mixed with other constituents, e.g. in order to increase the activity and/or shelf life. These constituents may be salts, pH buffers, stabilizers (such as skimmed milk or casein hydrolysate), emulsifiers adjuvants to improve the immune response (e.g. oils, muramyl dipeptide, aluminiumhydroxide, saponin, polyanions and amphipatic substances) and preservatives.

It is clear that a vaccine according to the invention may also contain immunogens related to other pathogens of poultry or may contain nucleic acid sequences encoding these immunogens, like antigens of Infectious Bronchitis Virus (IBV), Newcastle Disease Virus (NDV), Infectious Bursal Disease Virus (IBDV) or Marek's Disease Virus different from those disclosed herein, to produce a multivalent vaccine.

The invention also relates to an "immunochemical reagent", which reagent comprises a polypeptide according to the invention.

The term "immunochemical reagent" signifies that the polypeptide according to the invention is bound to a suitable support or is provided with a labelling substance.

The supports which can be used are, for example, the inner wall of a microtest well or a cuvette, a tube or capillary, a membrane, filter, test strip or the surface of a particle such as, for example, a latex particle, an erythrocyte, a dye sol, a metal sol or metal compound as sol particle.

Labelling substances which can be used are, inter alia, a radioactive isotope, a fluorescent compound, an enzyme, a dye sol, metal sol or metal compound as sol particle.

Example 1

Origin of Colorado isolate of APV

Swabs were used to collect material from infected turkeys, which material was then passaged 3 times via the yolk sac of embryonated chicken eggs followed by several passages in CEF (Senne, D. A. *et al*, Proc. Amer. Vet. Med. Assoc. 134th Annual Congress, Reno, Nevada, July 1997, p190). The turkey swab material was inoculated via the yolk sac into embryonated eggs. After 9 days, fluid collected from these embryos was inoculated "blind" into further embryos, again via the yolk sac. These embryos were incubated for a further 9 days, at which time haemorrhages were observed on the embryos and some mortality was seen. Harvested material was then inoculated onto CEF cultures and three passages made at 7 day intervals. The cytopathic effect seen was characterised by syncytial and giant cell formation. Preliminary characterisation as a pneumovirus was done using a TRT fluorescent antibody.

Cloning of the viral F gene

Chicken embryo fibroblasts ($2x10^7$) were infected with one ampoule of the Colorado pneumovirus isolate and incubated at 37°C. 48-72 hours later mRNA was isolated from these cells using the polyAtract 1000 kit (Promega) according to manufacturer's instructions. This mRNA was then subjected to RT-PCR using the Access RT-PCR system from Promega using an oligo-dT primer and a primer partially based on TRT gene sequences. The cycles of the RT-PCR were performed as follows:

One cycle at 48°C for 45 minutes.
One cycle at 94°C for 3 minutes.
40 cycles at 94°C for 30 seconds, 30°C for 30 seconds, and 68°C for 2 minutes.
One cycle at 68°C for 7 minutes.

The PCR reaction was electrophoresed on a 0.8% agarose gel, a 1.6kb fragment excised and then gel purified using the Qiagen gel purification kit according to manufacturers

instructions. This fragment was ligated into the pGEM T -easy vector (Promega) using the pGEM T-easy vector system (Promega) according to manufacturers instructions.

Following overnight ligation at 14°C the ligation mixture was cleaned using the Qiagen PCR purification kit according to manufacturers instructions, resuspended in 2µl of H_2O and electroporated into electrocompetent DH5α bacteria at 2.5kV, 200ohms, 25µF. The bacteria were then shaken at 37°C for one hour in 1ml LB broth. Following this 0.5ml of the broth was added to a LB plate containing 100µg/ml ampicillin, 0.5mM IPTG and 80µg/ml X-gal. Following overnight incubation at 37°C, 20 white colonies were picked and added to 1.5ml of LB broth containing 100µg/ml ampicillin and shaken overnight. Plasmid DNA was prepared from these cultures using standard methods and digested with Not1 enzyme. Plasmid DNA that contained a 1.6kb insert fragment was selected for sequencing and clones called F/pGEM.

Example 2

DNA sequence analysis

Clones of F/pGEM were grown in 100ml of Luria Bertani broth containing 100µg/ml ampicillin overnight with shaking at 37^0C. Plasmid DNA was isolated using a Qiagen maxi-prep kit according to manufacturers instructions. Samples of plasmid DNA were sent for ABI sequencing by Pat Barker at the Microchemical Facility in Babraham Institute, Babraham, Cambridge. The complete sequence of the clones was determined on each strand. Sequence data was analysed at the Elms using the Omiga software program (Oxford Molecular Ltd).

Example 3

Expression of the F protein

For analysis of the protein the F gene was sub-cloned by standard methods into the multiple cloning site of the pUC-derived mammalian expression vector pI.18 (obtainable from

Dr. Jim Robertson, NIBSC, South Mimms, EN6 3QG, UK). The F gene sequence was digested from the F/pGEM vector using the Not 1 enzyme, the fragment gel-purified, blunted using T4 DNA polymerase and ligated into pI.18 vector that had been digested with the EcoRV enzyme and treated with calf intestinal alkaline phosphatase. Orientation of the insert was determined by digestion with restriction enzymes. Insertion of the gene of interest into the multiple cloning site of this vector brings it under the transcriptional control of the human CMV promoter and intron A sequence. Transcription is terminated by the hCMV poly A signal. Expression of the F protein was detected by transfecting secondary chicken embryo fibroblasts with the F/pI.18 plasmid by electroporation and incubating the cells in M6B8 medium containing 2% foetal calf serum for 48 hours at 37°C/5%CO_2. The cells were fixed in 70% acetone, air dried and stained with antisera raised in chickens specific for the Colorado isolate.

Example 4

DNA vaccination of turkeys with a plasmid expressing the F protein of the Colorado isolate of avian pneumovirus

Experimental Design

Three groups of ten 5-day-old turkey poults, from hens in which no TRT vaccine has been used were formed. Two of the groups were immunised via the intramuscular route with plasmid DNA. Group one was immunised with 100μg of ttheF/pI.18 plasmid and group 2 with 100μg of the control pI.18 plasmid. Group 3 was left uninoculated. Ten siblings were bled. By 3 weeks of age the poults were wing banded, each with a unique number.

The poults were bled three and approximately six weeks after inoculation. Three weeks after inoculation the poults were re-vaccinated as above. At six weeks of age all 3 groups were challenged wit ha virulent strain of APV. At 10 days post challenge, the birds were bled and the experiment terminated. Serum samples were tested by ELISA for antibodies to the Colorado isolate of APV.

Results

Ten siblings were bled at 5 days of age and the sera tested by ELISA for antibodies to the Colorado isolate of APV. All had titres of $<\log_2 4.6$.

The poults were bled prior to the second vaccination (26 days of age) and prior to challenge at 6 weeks of age (2 weeks after the second vaccination). The results are shown in Table 1.

Table 1

APV ELISA titres (GMT) in poults at 6 weeks of age and post challenge.

Group	APV antibody titre ($\log_2$)	
	6 weeks	Post challenge
F/pI.18	5.2	14.5
PI.18	<4.6	11.1
Challenge control	<4.6	10.7

Prior to challenge at 6 weeks of age, an antibody response to the Colorado isolate was seen in 5/8 of the poults vaccinated with the F/pI.18 plasmid. All poults in the other two groups were still negative at that time. By 10 days post challenge, an antibody response was detected in all three groups. It was significantly higher in the group given the plasmid containing the APV F gene than in either the group given the parent plasmid or in the unvaccinated group ($p<0.001$ in each case).

CLAIMS

1. A nucleic acid sequence encoding a polypeptide comprising at least part of the protein expressed by the F gene of an avian pneumovirus characterised in that the polypeptide has an amino acid sequence shown in SEQ. ID. No. 2 or is a functional variant thereof.

2. A nucleic acid sequence according to claim 1 characterised in that the polypeptide exhibits at least 95% homology with the amino acid sequence shown in SEQ. ID. No. 2.

3. A recombinant nucleic acid molecule comprising a nucleic acid sequence according to claim 1 or claim 2.

4. A recombinant nucleic acid molecule according to claim 3 characterised in that the nucleic acid sequence is operably linked to an expression control sequence.

5. A recombinant vector virus containing the heterologous nucleic acid sequence according to claim 1 or claim 2.

6. A host cell transformed with a nucleic acid sequence according to claim 1 or claim 2 or with a recombinant nucleic acid molecule according to claim 3 or 4 or containing a recombinant vector virus according to claim 5.

7. A polypeptide with the amino acid sequence of the F protein of an avian pneumovirus having the amino acid sequence shown in SEQ. ID. No. 2 or a fuctional variant thereof.

8. A polypeptide according to claim 7 characterised in that it exhibits at least 95% homology with the amino acid sequence shown in SEQ. ID. No. 2.

9. An avian pneumovirus related polypeptide encoded by a nucleic acid sequence according to claim 1 or claim 2.

10. A process for expressing the polypeptide according to any one of claims 7 to 9 comprising culturing a host cell according to claim 6.

11. An antibody or antiserum immunoreactive with a polypeptide according to any of the claims 7 to 9.

12. A vaccine for the protection of poultry against avian pneumovirus characterised in that it comprises a recombinant vector virus according to claim 5, a host cell according to claim 6, a polypeptide according to any one of claims 7 to 9 or a polypeptide prepared by the process according to claim 10, together with an acceptable carrier.

13. A method for the preparation of an avian pneumovirus vaccine, characterised in that a host cell according to claim 6 is cultured, whereafter avian pneumovirus containing material is collected and processed to a pharmaceutical preparation with immunizing activity.

14. A method for the protection of poultry against avian pneumovirus infection characterised in that an effective amount of a vaccine according to claim 12 is administered to the animals.

15. An immunological reagent comprising a polypeptide according to any one of claims 7 to 9.

16. A test kit for carrying out an immuno-assay comprising an immunochemical reagent according to claim 15.

SEQUENCE LISTING

<110> AKZO Nobel N.V.

<120> Avian pneumovirus vaccine & diagnostic agent

<130> APV

<140>
<141>

<150> EP 98203352.4
<151> 1998-10-05

<160> 2

<170> PatentIn Ver. 2.1

<210> 1
<211> 1614
<212> DNA
<213> Avian pneumovirus

<220>
<221> CDS
<222> (1)..(1611)

<400> 1

```
atg tct tgg aaa gtg gta ctg cta ttg gta ttg cta gct acc cca acg   48
Met Ser Trp Lys Val Val Leu Leu Leu Val Leu Leu Ala Thr Pro Thr
  1               5                  10                  15

ggg ggg cta gaa gaa agt tat cta gag gag tca tgc agt act gtt act   96
Gly Gly Leu Glu Glu Ser Tyr Leu Glu Glu Ser Cys Ser Thr Val Thr
              20                  25                  30

aga gga tac ctg agt gtt ttg agg aca gga tgg tat aca aat gtg ttc   144
Arg Gly Tyr Leu Ser Val Leu Arg Thr Gly Trp Tyr Thr Asn Val Phe
          35                  40                  45

aca ctt gag gtt gga gat gtg gaa aat ctc aca tgt acc gac ggg ccc   192
Thr Leu Glu Val Gly Asp Val Glu Asn Leu Thr Cys Thr Asp Gly Pro
      50                  55                  60

agc tta ata aga aca gaa ctt gaa ctg aca aaa aat gca ctt gag gaa   240
Ser Leu Ile Arg Thr Glu Leu Glu Leu Thr Lys Asn Ala Leu Glu Glu
  65                  70                  75                  80

ctc aag aca gta tca gca gat caa ttg gca aag gaa gct agg ata atg   288
Leu Lys Thr Val Ser Ala Asp Gln Leu Ala Lys Glu Ala Arg Ile Met
                  85                  90                  95
```

```
tca cca aga aaa gcc cgg ttt gtt ctg ggt gcc ata gca tta ggt gtg   336
Ser Pro Arg Lys Ala Arg Phe Val Leu Gly Ala Ile Ala Leu Gly Val
            100                 105                 110

gca act gct gct gct gtg acg gct ggt gta gcg ata gcc aag aca att   384
Ala Thr Ala Ala Ala Val Thr Ala Gly Val Ala Ile Ala Lys Thr Ile
        115                 120                 125

agg cta gaa gga gaa gtg gct gca atc aag ggt gcg ctc agg aaa aca   432
Arg Leu Glu Gly Glu Val Ala Ala Ile Lys Gly Ala Leu Arg Lys Thr
    130                 135                 140

aat gag gct gta tct aca tta gga aat ggc gtg agg gta ctt gca aca   480
Asn Glu Ala Val Ser Thr Leu Gly Asn Gly Val Arg Val Leu Ala Thr
145                 150                 155                 160

gct gtg aat gat ctc aag gac ttt ata agt aaa aaa ttg aca cct gca   528
Ala Val Asn Asp Leu Lys Asp Phe Ile Ser Lys Lys Leu Thr Pro Ala
                165                 170                 175

ata aac agg aac aag tgt gac atc tca gac ctt aag atg gca gtg ggc   576
Ile Asn Arg Asn Lys Cys Asp Ile Ser Asp Leu Lys Met Ala Val Gly
            180                 185                 190

ttt gga caa tac aat cgg agg ttc ctc aat gtg gta aga cag ttt tct   624
Phe Gly Gln Tyr Asn Arg Arg Phe Leu Asn Val Val Arg Gln Phe Ser
        195                 200                 205

gac aat gca ggt att acg cct gca ata tct cta gat tta atg act gac   672
Asp Asn Ala Gly Ile Thr Pro Ala Ile Ser Leu Asp Leu Met Thr Asp
    210                 215                 220

gct gag ctt gta aga gct gta agc aac atg ccc aca tct tca gga cag   720
Ala Glu Leu Val Arg Ala Val Ser Asn Met Pro Thr Ser Ser Gly Gln
225                 230                 235                 240

atc aat ctg atg ctt gag aat cgg gca atg gtc aga agg aaa gga ttt   768
Ile Asn Leu Met Leu Glu Asn Arg Ala Met Val Arg Arg Lys Gly Phe
                245                 250                 255

ggg att ttg att gga gtt tat ggt agc tct gtg gtc tat ata gtg cag   816
Gly Ile Leu Ile Gly Val Tyr Gly Ser Ser Val Val Tyr Ile Val Gln
            260                 265                 270

ctt cct att ttc ggt gtg ata gat aca ccg tgt tgg aag gtg aag gct   864
Leu Pro Ile Phe Gly Val Ile Asp Thr Pro Cys Trp Lys Val Lys Ala
        275                 280                 285

gct cca tta tgt tca ggg aaa gac ggg aat tat gca tgt ctc ttg cga   912
Ala Pro Leu Cys Ser Gly Lys Asp Gly Asn Tyr Ala Cys Leu Leu Arg
    290                 295                 300

gag gac caa ggt tgg tat tgt caa aat gct gga tcc aca gtt tat tat   960
```

```
Glu Asp Gln Gly Trp Tyr Cys Gln Asn Ala Gly Ser Thr Val Tyr Tyr
305                 310                 315                 320

cca aat gag gag gac tgt gaa gta aga agt gat cat gtg ttt tgt gac   1008
Pro Asn Glu Glu Asp Cys Glu Val Arg Ser Asp His Val Phe Cys Asp
                325                 330                 335

aca gca gct ggg ata aat gta gca aag gag tca gaa gag tgc aac agg   1056
Thr Ala Ala Gly Ile Asn Val Ala Lys Glu Ser Glu Glu Cys Asn Arg
            340                 345                 350

aat atc tca aca aca aag tac cct tgc aag gta agt aca ggg cgt cac   1104
Asn Ile Ser Thr Thr Lys Tyr Pro Cys Lys Val Ser Thr Gly Arg His
        355                 360                 365

cca ata agc atg gtg gcc tta tca cca ctg ggt gct ttg gta gcc tgt   1152
Pro Ile Ser Met Val Ala Leu Ser Pro Leu Gly Ala Leu Val Ala Cys
    370                 375                 380

tat gac ggt atg agt tgt tcc att gga agc aac aag gtt gga ata atc   1200
Tyr Asp Gly Met Ser Cys Ser Ile Gly Ser Asn Lys Val Gly Ile Ile
385                 390                 395                 400

aga cct ttg ggg aaa ggg tgt tca tac atc agc aat caa gat gct gac   1248
Arg Pro Leu Gly Lys Gly Cys Ser Tyr Ile Ser Asn Gln Asp Ala Asp
                405                 410                 415

act gtt aca att gac aac aca gtg tac caa ttg agc aaa gtt gaa gga   1296
Thr Val Thr Ile Asp Asn Thr Val Tyr Gln Leu Ser Lys Val Glu Gly
            420                 425                 430

gaa caa cac aca att aaa ggg aag cca gta tct agc aat ttt gac cct   1344
Glu Gln His Thr Ile Lys Gly Lys Pro Val Ser Ser Asn Phe Asp Pro
        435                 440                 445

ata gag ttc cct gaa gat cag ttc aac ata gcc ctg gat cag gtg ttt   1392
Ile Glu Phe Pro Glu Asp Gln Phe Asn Ile Ala Leu Asp Gln Val Phe
    450                 455                 460

gaa agt gtt gag aag agt cag aat ctg ata gac cag tca aac aag ata   1440
Glu Ser Val Glu Lys Ser Gln Asn Leu Ile Asp Gln Ser Asn Lys Ile
465                 470                 475                 480

ttg gat agc att gaa aag ggg aat gca gga ttt gtc ata gtg ata gtc   1488
Leu Asp Ser Ile Glu Lys Gly Asn Ala Gly Phe Val Ile Val Ile Val
                485                 490                 495

ctc att gtc ctg ctc atg ctg gca gca gtt ggt gtg ggt gtc ttc ttt   1536
Leu Ile Val Leu Leu Met Leu Ala Ala Val Gly Val Gly Val Phe Phe
            500                 505                 510

gtg gtt aag aag aga aaa gct gct ccc aaa ttc cca atg gaa atg aat   1584
Val Val Lys Lys Arg Lys Ala Ala Pro Lys Phe Pro Met Glu Met Asn
        515                 520                 525
```

ggt gtg aac aac aaa gga ttt atc cct taa 1614
Gly Val Asn Asn Lys Gly Phe Ile Pro
530 535

<210> 2
<211> 537
<212> PRT
<213> Avian pneumovirus

<400> 2
Met Ser Trp Lys Val Val Leu Leu Leu Val Leu Leu Ala Thr Pro Thr
1 5 10 15

Gly Gly Leu Glu Glu Ser Tyr Leu Glu Glu Ser Cys Ser Thr Val Thr
20 25 30

Arg Gly Tyr Leu Ser Val Leu Arg Thr Gly Trp Tyr Thr Asn Val Phe
35 40 45

Thr Leu Glu Val Gly Asp Val Glu Asn Leu Thr Cys Thr Asp Gly Pro
50 55 60

Ser Leu Ile Arg Thr Glu Leu Glu Leu Thr Lys Asn Ala Leu Glu Glu
65 70 75 80

Leu Lys Thr Val Ser Ala Asp Gln Leu Ala Lys Glu Ala Arg Ile Met
85 90 95

Ser Pro Arg Lys Ala Arg Phe Val Leu Gly Ala Ile Ala Leu Gly Val
100 105 110

Ala Thr Ala Ala Ala Val Thr Ala Gly Val Ala Ile Ala Lys Thr Ile
115 120 125

Arg Leu Glu Gly Glu Val Ala Ala Ile Lys Gly Ala Leu Arg Lys Thr
130 135 140

Asn Glu Ala Val Ser Thr Leu Gly Asn Gly Val Arg Val Leu Ala Thr
145 150 155 160

Ala Val Asn Asp Leu Lys Asp Phe Ile Ser Lys Lys Leu Thr Pro Ala
165 170 175

Ile Asn Arg Asn Lys Cys Asp Ile Ser Asp Leu Lys Met Ala Val Gly
180 185 190

Phe Gly Gln Tyr Asn Arg Arg Phe Leu Asn Val Val Arg Gln Phe Ser
195 200 205

Asp Asn Ala Gly Ile Thr Pro Ala Ile Ser Leu Asp Leu Met Thr Asp
210 215 220

```
Ala Glu Leu Val Arg Ala Val Ser Asn Met Pro Thr Ser Ser Gly Gln
225                 230                 235                 240

Ile Asn Leu Met Leu Glu Asn Arg Ala Met Val Arg Arg Lys Gly Phe
                245                 250                 255

Gly Ile Leu Ile Gly Val Tyr Gly Ser Ser Val Val Tyr Ile Val Gln
            260                 265                 270

Leu Pro Ile Phe Gly Val Ile Asp Thr Pro Cys Trp Lys Val Lys Ala
        275                 280                 285

Ala Pro Leu Cys Ser Gly Lys Asp Gly Asn Tyr Ala Cys Leu Leu Arg
    290                 295                 300

Glu Asp Gln Gly Trp Tyr Cys Gln Asn Ala Gly Ser Thr Val Tyr Tyr
305                 310                 315                 320

Pro Asn Glu Glu Asp Cys Glu Val Arg Ser Asp His Val Phe Cys Asp
                325                 330                 335

Thr Ala Ala Gly Ile Asn Val Ala Lys Glu Ser Glu Glu Cys Asn Arg
            340                 345                 350

Asn Ile Ser Thr Thr Lys Tyr Pro Cys Lys Val Ser Thr Gly Arg His
        355                 360                 365

Pro Ile Ser Met Val Ala Leu Ser Pro Leu Gly Ala Leu Val Ala Cys
    370                 375                 380

Tyr Asp Gly Met Ser Cys Ser Ile Gly Ser Asn Lys Val Gly Ile Ile
385                 390                 395                 400

Arg Pro Leu Gly Lys Gly Cys Ser Tyr Ile Ser Asn Gln Asp Ala Asp
                405                 410                 415

Thr Val Thr Ile Asp Asn Thr Val Tyr Gln Leu Ser Lys Val Glu Gly
            420                 425                 430

Glu Gln His Thr Ile Lys Gly Lys Pro Val Ser Ser Asn Phe Asp Pro
        435                 440                 445

Ile Glu Phe Pro Glu Asp Gln Phe Asn Ile Ala Leu Asp Gln Val Phe
    450                 455                 460

Glu Ser Val Glu Lys Ser Gln Asn Leu Ile Asp Gln Ser Asn Lys Ile
465                 470                 475                 480

Leu Asp Ser Ile Glu Lys Gly Asn Ala Gly Phe Val Ile Val Ile Val
                485                 490                 495

Leu Ile Val Leu Leu Met Leu Ala Ala Val Gly Val Gly Val Phe Phe
            500                 505                 510
```

Val Val Lys Lys Arg Lys Ala Ala Pro Lys Phe Pro Met Glu Met Asn
515 520 525

Gly Val Asn Asn Lys Gly Phe Ile Pro
530 535

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/EP 99/07400

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/45 C07K14/115 C07K16/10 A61K39/155 G01N33/569

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	YU Q ET AL.: "Protection against turkey rhinotracheitis pneumovirus (TRTV) induced by afowlpox virus recombinant expressing the TRTV fusion glycoprotein (F)" VACCINE, vol. 12, no. 6, May 1994 (1994-05), pages 569-573, XP002095553 GUILDFORD GB cited in the application page 570 -page 571	1,3-7, 9-16

-/--

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 January 2000	28/01/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Cupido, M

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte nal Application No
PCT/EP 99/07400

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	NAYLOR C J ET AL.: "The ectodomains but not the transmembrane domains of the fusion proteins of subtypes A and B avian pneumovirus are conserved to a similar extent as those of human respiratory syncytial virus" JOURNAL OF GENERAL VIROLOGY, vol. 79, no. 6, June 1998 (1998-06), pages 1393-1398, XP002095552 READING GB figure 1	1,3-7,9, 10
X	YU Q ET AL.: "Deduced amino acid sequence of the fusion glycoprotein of turkey rhinotracheitis virus has greater identity with that of human respiratory syncytial virus, a pneomovirus, than that of paramyxoviruses and morbilliviruses" JOURNAL OF GENERAL VIROLOGY, vol. 72, no. 1, January 1991 (1991-01), pages 75-81, XP002095554 READING GB figure 2	1,3-7,9, 10
P,X	SEAL BS: "Matrix protein gene nucleotide and predicted amino acid sequence demonstrates that the first US avian pneomovirus isolate is distinct from European strains" VIRUS RESEARCH, vol. 58, no. 1-2, November 1998 (1998-11), pages 45-52, XP002096113 the whole document	1-10
X,P	Database EMBL Emvr1 Sequence ID AF085228 01-07-1999, Dar A. et al.:"Avian pneumovirus matrix protein (M) gene, partial cds; and fusion glycoprotein (F) gene, complete cds. XP002127916 & CHIANG SJ ET AL.: "Isolation of avian pneumovirus in QT-35 cells" VETERINARY RECORD, vol. 143, no. 21, 21 November 1998 (1998-11-21), page 596	1-4,7,8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 99/ 07400

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim 14
is directed to a method of treatment of the animal
body, the search has been carried out and based on the alleged
effects of the vaccine

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.